Analysis of the diversity of the intestinal microbiota of rats subjected to resection of the ileocecalvalve and creation of artificial sphincter

Análise da diversidade da microbiota intestinal de ratos submetidos à ressecção da valva ileocecal e criação de esfíncter artificial

Ângela Aparecida Barra, TCBC-MG[1]; Alcino Lázaro da Silva, ECBC-MG[2]; Cícero de Lima Rena, ECBC-MG[3]; Maria Cristina Vasconcelos Furtado[4]; Alessandra Barbosa Ferreira[5]; Marcelo Nagen[6]; Fernanda de Souza Freitas[7]

A B S T R A C T

Objective: To analyze, through molecular biology, the diversity of the intestinal microbiota before and after resection of the ileocecal junction and reconstruction of intestinal transit with and without the creation of a neosphincter. **Methods**: Fourteen Wistar rats were divided into two groups: Group A (n = 7), submitted to resection of the ileocecal valve and end-to-end, single-layer ileocolic anastomosis; and Group B (n = 7) with resection of the ileocecal valve and end-to-end, single-layer ileocolic anastomosis followed by construction of an artificial sphincter. Intraluminal contents were collected from both groups. The animals were reoperated 20 days after the first procedure, with new collection of intraluminal contents of the ileum and colon. From the samples collected, DNA was extracted for PCR-DGGE. The electrophoretic banding patterns generated in the reaction were analyzed for similarities and diversities of the microbiota. **Results**: The diversity of microorganisms was larger and in more samples when collected from the ileum than from the colon. The group with the neosphincter showed the highest variation in the colon, from 2.11 to 2.93. In three animals from each group w established similarity comparison, and they displayed no similarity with controls. **Conclusion**: ileocecal resection led to changes in ileal microbiota and, with the creation of new sphincter, the changes were even greater.

Key words: Intestines. Anal canal. Ileocecal valve. Polymerase chain reaction. Molecular biology.

INTRODUCTION

The ileocecal junction is a muscle structure with function of a sphincter[1]. It regulates the ileal flow to the cecum, contributing to the absorptive process. It hampers cecoileal reflux, reducing colonization with colonic bacteria in the ileum[2,3].

The anatomic and / or functional loss of the ileocecal region causes dysbiosis. It is a bacterial overgrowth of species outside their usual sites. The clinical manifestation of dysbiosis corresponds to malabsorptive syndrome[4,5].

The digestive tract is colonized by over 30,000 species of bacteria, and more than 60% uncultivable outside the intestinal environment by conventional techniques. With the use of new molecular biology techniques, such as DNA extraction and polymerase chain reaction (PCR), there has been successful identification of microorganisms, especially the uncultivable or those that did not survive the transport and storage techniques for cultivation[6-8].

The creation of artificial sphincters or valves in the intestine aims to reduce transit speed and prevent reflux of colonic contents into the small intestine. Several techniques have been described, such as external constriction, through suturing or ring, segmental intussusception of the bowel, submucosal tunneling, myectomies, seromyectomies or seromyotomies. These techniques are employed to reduce the clinical situations such as short bowel syndrome[3,9-15]. From the modification of the technique of Lázaro da Silva[16,17], Rena et al. created an artificial valve in 20 patients, with good results. They used two circumferential seromyotomies with a buried intermediate seromuscular ring, constituting a pylorus with preserved intrinsic innervation[18].

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1. Associate Professor, Department of Medicine and Nursing, Federal University of Viçosa; 2. Emeritus Professor, Federal University of Minas Gerais; 3. Assistant Professor, Federal University of Juiz de For a; 4. Associate Professor, Federal University of Juiz de Fora; 5. PhD, Agricultural Microbiology, Federal University of Viçosa; 6. MSc, Agricultural Microbiology, Federal University of Viçosa; 7. Biochemist, Federal University of Viçosa.

The purpose of this experimental study was to analyze molecular biology through the diversity of the ileocecal microbiota before and after ileocecal junction resection and reconstruction of transit with and without the creation of a "neosphincter."

METHODS

We used 14 male Wistar rats (Rattus norvegicus albinus) aged six months, from the vivarium of the Center for Biology of Reproduction (CBR), Federal University of Juiz de Fora. This center is registered with the Brazilian College of Animal Experimentation. The study was approved by the Ethics Committee on Animal Experiments of the Research Sub-rectory of the Federal University of Juiz de Fora under No 02/2010.

The animals were divided into two groups: Group A (n = 7) with resection of the ileocecal valve and single-layer, end-to-end ileocolic anastomosis; and Group B (n = 7) with resection of the ileocecal valve, end-to-end, single-layer ileocolic anastomosis and making of an artificial sphincter by the Rena et al. technique[18].

The animals were housed in polypropylene cages, with selected wood shavings, water bottle and chow trough. The cages were conditioned cabinets with controlled temperature, light, humidity and programmed air exchange. Preoperatively the animals fasted for six solid hours, but sugar water (5g/100ml) was given ad libitum. Once weighed, the animals were anesthetized. The anesthetic technique, identical between the groups, consisted of intraperitoneal injection of ketamine 10mg/kg, associated with ketamine. During the postoperative period, the animals were kept in cages and appropriate environment and received only water on the first day after surgery. Over the next two days, they received crushed chow soaked in water. On the fourth day the pasty chow was replaced by pelleted one[19].

In the first surgery, antisepsis was realized in the area of the abdomen with alcohol 70°GL. Under strict aseptic technique, we performed a median longitudinal incision of 3cm, in the skin and subcutaneous tissue. The muscles were separated in the median plane and the peritoneal cavity was reached; we then identified the ileocolic segment, which was exteriorized from the peritoneal cavity. The ileocecocolic vascular pedicle was ligated with nonabsorbable suture (Nylon 4.0), along the mesenteric side. Next, the terminal ileum was sectioned 2cm from the ileocecal junction to collect the enteric content in a sterile tube. The amount collected was dependent on the content that was there. We resected the ileal segment according to the demarcated ischemia, as well as the cecum and 2cm of the ascending colon. From the colonic extremity we also collected intraluminal content at the approximate volume of the ileum, reserved on another sterile tube (Figure 1A). The tubes were identified with the segment of origin and number of the animal, and stored under refrigeration at -4° C.

In group A we performed an end-to-end ileocolic anastomosis and closure of the cavity (Figure 1 B).

In group B, after termino-terminal anastomosis, we constructed an artificial sphincter according to the technique described by Rena et al.[18], 5cm proximal to the anastomosis, provided that, between the distal and proximal seromyotomies, the seromuscular ring was 2mm long (Fig. 2 A, B, C and D).

The animals were followed for a period of 20 days, then undergoing the second surgery. Under the same anesthetic technique and dose, they were positioned for opening of the peritoneal cavity and release of adhesions. The region involving the ileocolic anastomosis was exposed to enterotomy and sampling of the intraluminal contents of the colon and pre-anastomosis ileal segment in groups A and B. Samples were also collected in the intermediate segment between the anastomosis and the valve in group B, under the same conditions of strict aseptic technique (Figure 3A and B).

Samples of intraluminal content, collected with spatula and sterile tubes, were identified by individual animal number and the segment from which they were

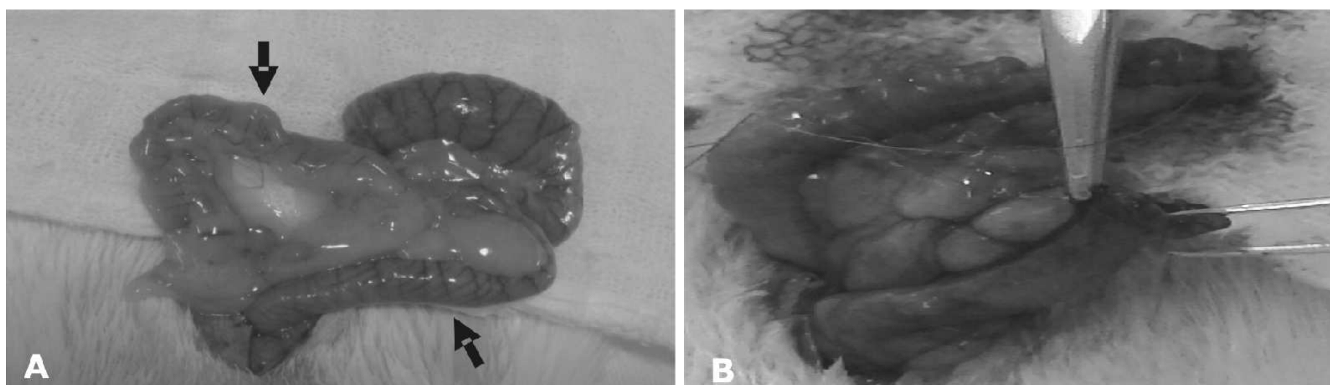


Figure 1 – *A: Location in the ileocecocolic segment where the intestinal intraluminal content was collected (arrows), B: Beginning of end-to-end ileocolic anastomosis.*

acquired and were kept in refrigeration at -4º C. Later, they were transferred to the Laboratory of Industrial Microbiology, Department of Microbiology at the Institute of Applied Agricultural Biotechnology of the Federal University of Viçosa, for molecular analysis of microbiota using PCR-DGGE.

The control sample was gathered from the withdrawal and transfer of the volume of 0.02g of the contents from the segments of ileum and colon of the seven animals in each group, at the first intervention, added in another tube, totaling four samples. From the enteric contents of each tube from the second intervention, a

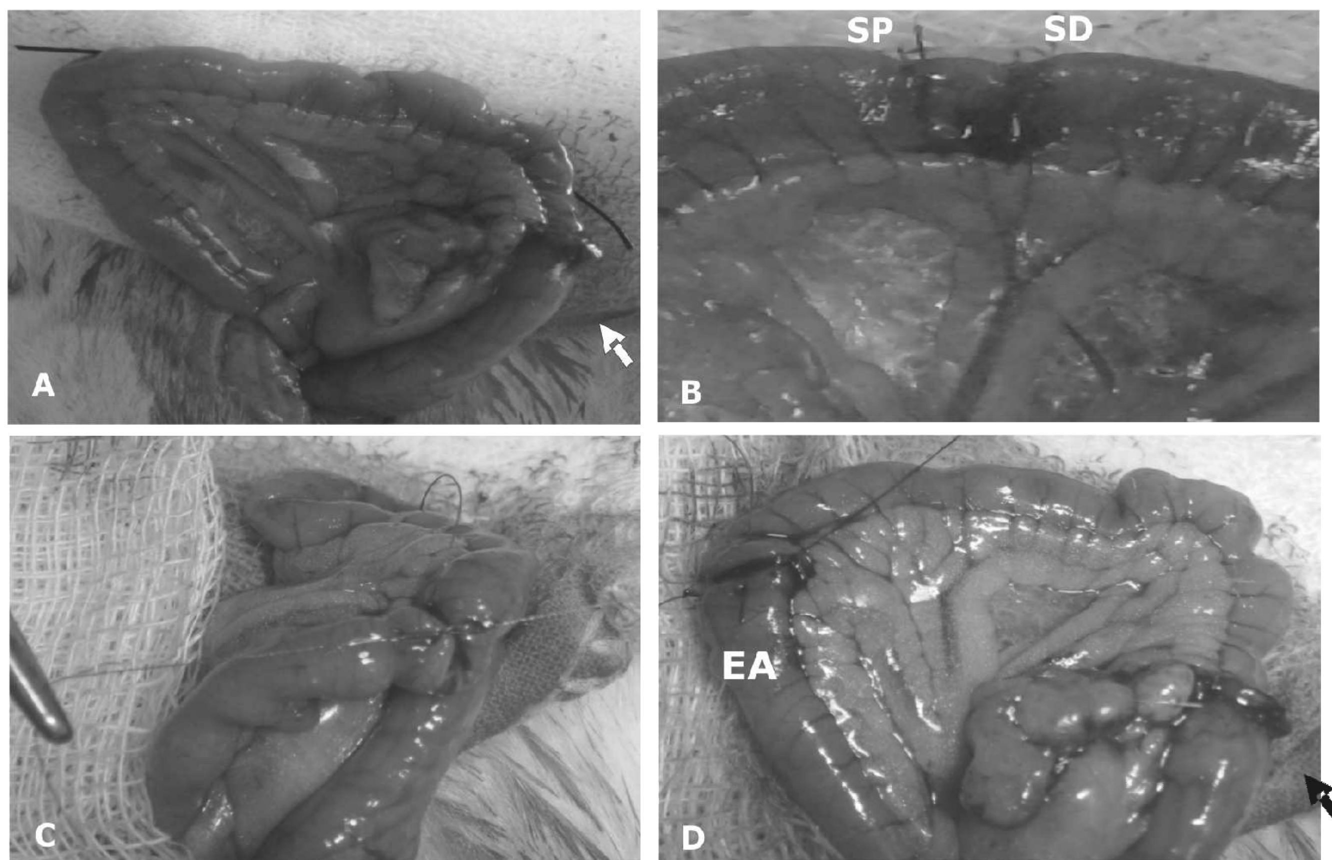
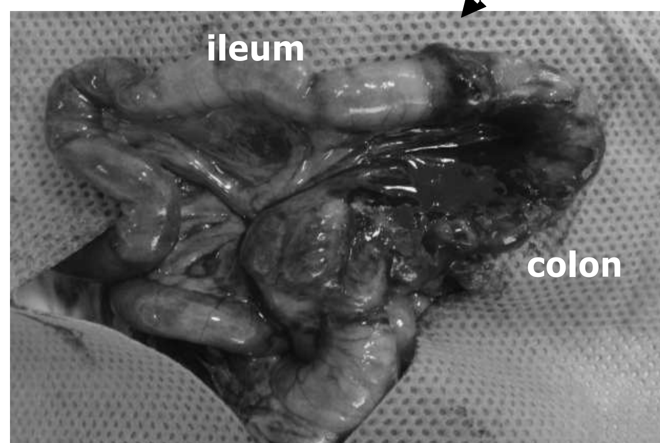


Figure 2 – *A: marking the site of seromyotomies with silk sutures 5cm from the anastomosis; B: 2mm seromuscular ring between the proximal SP and distal SD seromyotomies; C: suture, with separate stiches, of the distal edge of the distal seromiotomy to the proximal edge of the proximal seromiotomy, D: completion of the sphincter (EA) and anastomosis (arrow).*



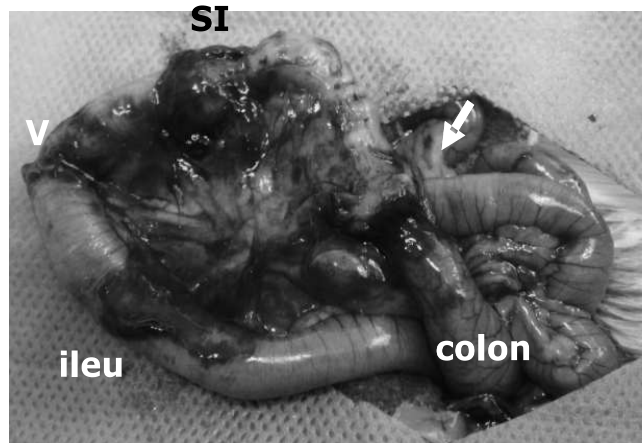


Figure 3 - *Representation of the region where the Intraluminal content sample was collected: anastomosis (arrow), valve (V), intermediate segment (SI).*

sample of 0.150 g was separated for each segment, generating 35samples. Thus, 39 samples were obtained for DNA extraction, distributed according to the group, animal and the segment of the collection of intraluminal content.

Total bacterial DNA was extracted from 39 samples using the QIAamp DNA Stool Mini kit. The extracted DNA samples were analyzed in 0.8% agarose gel in 1X TAE buffer [40 mM Tris-HCl, 20 mM acetic acid and 1mM EDTA (pH 8.3)]. After electrophoresis, the gel was kept under stirring in a solution of ethidium bromide (0.25 ìgmL^{-1})[20]. Quantitation was held in Nanodrop 2000. The extracted DNA was subjected to visualization in an image digitalization system.

From the DNA extracted, gene fragments encoding eubacterial rRNAs16S were amplified by polymerase chain reaction using specific primers 1392R and 27F and reaction conditions[21] (Table 1). The amplicons resulting from PCR amplification were analyzed on 1.2% agarose gel TAE buffer (40 mM Trisacetate and 1mM EDTA). After electrophoresis, the gel was kept under stirring in a solution of ethidium bromide (0.25 ìgmL^{-1})[20]. A size marker 100bp DNA ladder was used to estimate the sizes of the different amplicons[21].

Using a thermal cycler and nested primers containing the GC clamp (1378R and 984)[22] (Table 2), PCR-DGGE was performed in 200ìL tubes to a final volume of 25ìl. The reaction mixture was prepared using 5ìl of buffer GoTaq® Reaction Buffer (Promega, Madison, USA), 1.5 mM magnesium chloride (MgCl2) (Promega, Madison, USA) 200ìM of desoxirribo-nucleotide-triphosphate, 0.2 mM of each oligonucleotide primer, 5ìg of bovine serum albumin (BSA), 2% (v / v deionized formamide, 1.5 U Taq DNA polymerase, 20ng total DNA and sterile water to complete the final volume). For the separation of different amplicons without DGGE we applied 18ìL of PCR products in vertical polyacrylamide gel in TAE buffer, and the gradient of urea / formamide from 40% to 60%. For the separation of amplicons from the first PCR reaction we used polyacrylamide gel 6% (p/v), and for the amplicons of the second reaction, polyacrylamide gel 8% (p/v). Electrophoresis was conducted at a temperature of 60°C and constant voltage of 60V for 16 hours, and the gel stained with SYBR® Gold's. Electrophoreses were made in a equipment "DCode ™ Universal Mutation Detection System" (Bio-Rad - California USA).

Statistical comparison of the profiles of the gels bands of the 39 samples obtained after DGGE was performed using BioNumerics program. The variable "total bacteria" was estimated based on a binary array, wherein the presence of the band corresponding to each operational taxonomic unit (UTO) was coded as "1" and the absence as "0". The microbial community structure was evaluated based on the "Dice" similarity coefficient and the UPGMA method (Unweighted Pair Group Method with Arithmetic) for analyzing clusters. The data obtained with the aid of BioNumerics program were used to calculate the richness, the Shannon-Weaver diversity index (H), and evenness (E). The richness represents the number of bands in the PCR-DGGE gel, which refers to the operational taxonomic units (UTOs). The diversity index (H) is calculated as: H = 2.3/ N(NlogN-$\sum \mathrm{ni} \log \mathrm{ni}$) where N = sum of the mass of all DNA bands and ni = the mass of DNA band. The evenness (E) is expressed as E = H/logR, where H is the diversity index and R is the number of bands. The "Shannon-Weaver" is the general diversity index that increases with the number of species and is greatest when the mass of individuals is more evenly distributed among species. The evenness indicates whether there are dominant bands.

Table 1 - Process conditions for amplification of DNA extracted from samples.

Primers	Sequence	Conditions of reaction - 30 cycles		
		Denaturation	Girdling	Extension
1392R	5'-ACGGGCGGTGTGTAC3'	1 minute at 94°	1 minute at 54°	1 minute and 30 seconds at 72°
27F	5'-AGAGTTTGATCCTGGCTCAG3'			

Table 2 - Process conditions of PCR-DGGE.

Primers	Sequence	Reaction conditions - Initial temperature of 95 ° / 3 minutes 35 cycles		
		Denaturation	Girdling	Extension
F984GC	5'CGCCCGGGGCGCGCCCCGGG CGGGGCGGGGGCACGGGGG GAACGCGAAGAACCTTAC3	30 seconds at 94°	30 seconds at 57°	1 minute and 30 seconds a 72° Final extension /7 minutes
R1378	5'CGGTGTGTACAAGGCCCGGGAACG3			

RESULTS

In multivariate analysis of bacterial community structure arranged in dendrogram similarity "Dice", in only three animals of each group there was the grouping of bands in electrophoresis segments when compared to the control samples. In Group A samples from animals "3", "4" and "5" grouped, as well as samples from colonic and ileal segments of the controls. Similarity analyzed by the Dice coefficient dendrogram, considering samples of ileal and colonic segments, was 65%, 75% and 80% in animals "5", "3" and "4", respectively. In samples from the control group the similarity between the segments was 45% and were external to the groupings of the three animals in group A (Figure 4).

In group B the profile of the grouping bands of samples from ileal, intermediate and colonic segments in animal "5", "7" and "9" allowed comparative analysis with control samples. The similarity was 80% between colonic and intermediate segments and 62% in the segment of ileum in animal "5". In animal "7" it was 70% for the intermediate and ileal segments and 66% for the colon. In animal "9" it was 56% between colonic and ileal segments and 54% for the intermediate. As for the control the similarity of the segments was 55% and this was also separated from those of the other animals (Figure 5).

From the electrophoresis of the samples, we calculated values for diversity, evenness and richness. Among samples from the same animal for variable diversity, higher values were found in seven ileal samples, five samples from the colonic segment showed higher values and in three comparisons were very close. However, the highest value found corresponded to the diversity of colonic segment of animal "6" in group A. The lowest value found corresponded to the colonic segment of animal "9" in group B. For the attribute evenness, greater values were found in eight samples from the colon, close values in six control samples, and in two from the ileum values were higher. The highest value of evenness, 2.94, was found in the colonic sample of animal 4A, and the smallest, 2.23i, in the ileal sample from animal 7B. The richness ranged from 6UTOs to 18UTOs and extreme values were from samples of the colon of animals belonging to group B. In group A, the ileum control sample showed higher diversity when compared to the colon segment. The average diversity of post-intervention samples was lower than the controls' for the ileal segment, and higher for the colonic one. In Group B, the average diversity was greater in the postoperative period for both segments (Tables 3 and 4).

DISCUSSION

The creation of sphincter mechanisms in the small intestine is related to the control of intestinal transit speed, particularly in bowel resections. Studies in rats, dogs and humans has shown that artificial valves slow intestinal transit and promote weight gain after intestinal resections[18,23-26]. Besides these results, some described techniques show reduction of colonization of the proximal segments of the small intestine through the identification of bacterial growth by intraluminal collections or stool culture. The method has the limitation of not covering the universe of the intestinal microbiota, for cultivable species represent 20% to 40% of existing bowel bacteria[3,10,11,15,16].

The use of a molecular biology method with higher specificity and sensitivity have revealed more information about this ecosystem[21]. In the experiment carried out after confirmation of DNA extraction, amplification of the gene fragments was performed by PCR-DGGE to establish the diversity of microbiota in the different samples. The PCR-DGGE technique is used in studies of microbial ecology with representative results in the identification of species and abundance of different phylotypes of samples, and allows reliable estimates of microbial diversity[27,28].

The comparative arrangement of similarity of the samples with the control sample, in three of the seven animals, reveals that the intervention, both in the group in which was the ileocecal valve was removed and in the one

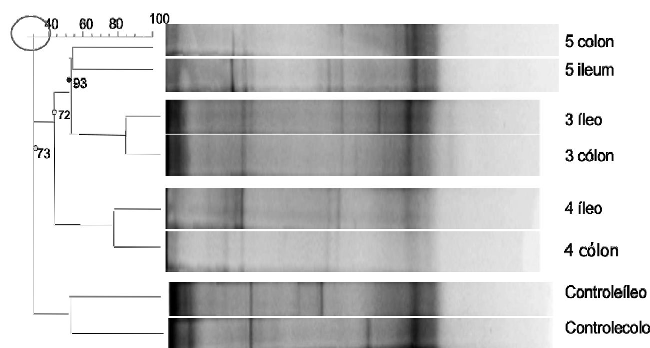


Figure 4 - *DGGE electrophoretic profiles of fragments of rDNAs 16S from the total bacteria in samples of colonic and ileal segments of animals 5, 3, and 4 and control from group A.*

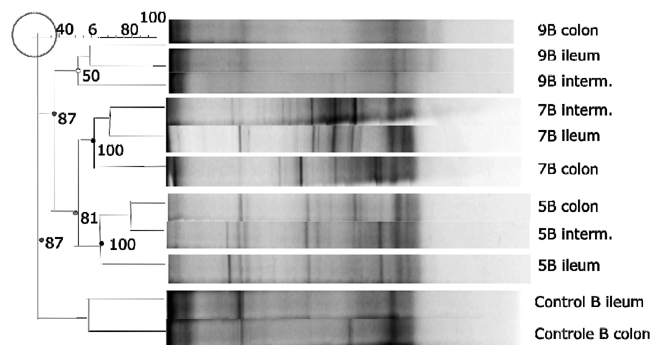


Figure 5 - *DGGE electrophoretic profiles of fragments from the rDNAs 16S of total bacteria in samples of ileal, intermediate colonic segments in animals 9, 7 and 5 and control in group B.*

Table 3 - Values of variables in samples from group A to all segments.

	"Shannon Weaver" H' Index		Evenness		Richness	
Control	Ileum	Colon	Ileum	Colon	Ileum	Colon
samples A	2.65	2.36	2.45	2.47	12	9
1ª	2.57	2.67	2.31	2.33	13	14
3ª	2.50	2.34	2.40	2.46	11	9
4ª	2.71	2.49	2.51	2.94	12	7
5ª	2.68	2.66	2.40	2.39	13	13
6ª	2.77	2.93	2.30	2.44	16	16
7ª	2.19	2.59	2.59	2.87	7	8
8ª	2.70	2.71	2.43	2.37	13	14
Mean	2.59	2.63	2.42	2.54	12.14	11.57

Note the highest circulated values

Table 4 - Values of variables in group B samples for all segments.

	Shannon Weaver H' Index		Evenness		Richness	
Control	Ileum	Colon	Ileum	Colon	Ileum	Colon
samples B	2.38	2.39	2.50	2.50	9	9
3B	2.64	2.54	2.37	2.44	13	11
4B	2.63	2.61	2.44	2.50	12	11
5B	2.89	2.69	2.35	2.49	17	12
6B	2.69	2.92	2.42	2.37	13	17
7B	2.62	2.82	2.23	2.25	15	18
8B	2.40	2.30	2.84	2.55	7	8
9B	2.37	2.11	2.49	2.72	9	6
Média	2.61	2.57	2.45	2.47	12.28	11.85

Note the lowest values

in which, in addition to resection, an "artificial sphincter," was created, the difference was 45%, indicating that the intervention may contribute to the modification of the diversity of total bacteria. The high diversity in most ileal samples of various animals, regardless of group, leads us to think that the methods so far used for this analysis underestimated this fact or, even the colon having a higher concentration of micro-organisms, the diversity of genera and species identified may be less. The significant difference of the diversity found in the sample of the ileal segment in group B when compared to the preoperative period requires further research to define whether this event was due to the rise of colonic bacteria into the ileum. Another issue to be investigated is related to decreased intestinal transit speed with the creation of the new "sphincter" contributing to modify the profile of the microbiota.

There still remains the challenge of addressing the ileocecal junction to investigate the microbiota. Not only anatomical loss, but the ileocecal junction dysfunction, can also evolve with changes in the distribution of microbiota. Machado et al., in a retrospective study with barium enema, identified 25% prevalence of reflux from the colon to the ileum, independent of age and gender[29]. What are the consequences of these changes? Developing strategies and alternatives to investigate the intestinal microbiota is extremely important, given that today it is considered a functionally active organ, capable of promoting health[28,30]. Research on the use of pre and pro-biotics have shown satisfactory results for conditions such as inflammatory bowel disease, obesity and colon cancer[4]. Nevertheless, it must be recognized that the intestinal microbiota presents peculiar behavior, described as a form of biometrics, and even clinical, nutritional and surgical interventions should be investigated thoroughly to offer greater subsidies for future research[7]. In this line of thought, this work aims to elucidate the findings of diversity through sequencing to identify possible bacterial genera and species from extracted DNA, besides its quantification.

In conclusion, the diversity of ileal microorganisms found was greater than that of the colon. The creation of an artificial sphincter increased this diversity.

R E S U M O

Objetivo: *analisar através de biologia molecular a diversidade da microbiota da junção ileocecal antes e após a ressecção da válvula ileocecal e reconstrução do trânsito com e sem a criação de "neoesfíncter".* ***Métodos***: *Os animais foram distribuídos em dois grupos: Grupo A (n=7) com ressecção da válvula ileocecal e anastomose ileocólica término-terminal em plano único, e Grupo B (n=7) com ressecção da válvula ileocecal e anastomose ileocólica término-terminal em plano único e confecção do esfíncter artificial. Reoperados com 20 dias coletou-se novamente conteúdo intraluminar do íleo e do cólon. Das amostras coletadas, extraiu-se DNA para reação de PCR-DGGE. Os padrões de bandas eletroforéticas , gerados na reação, foram submetidos ao programa Bionumerics para análise da similaridade e da diversidade da microbiota.* ***Resultados***: *a diversidade da microbiota foi maior e em mais amostras do íleo do que as do cólon. O grupo com a válvula apresentou os maiores valores e variações no cólon de 2,11 a 2,93. Em três animais de cada grupo estabeleceu-se comparação da similaridade e não se assemelharam ao controle.* ***Conclusão:*** *a ressecção da válvula ileocecal levou à mudanças da microbiota ileal e, com a criação de novo esfíncter, as variações foram maiores.*

Descritores: *Intestinos. Canal anal. Valva ileocecal. Reação em cadeia da polimerase. Biologia molecular.*

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Address for correspondence:
Angela Barra Aparecida
E-mail: barra.angela@oi.com.br